TITLE OF THE INVENTION

GENETIC SEQUENCES ENCODING SUBSTRATE-SPECIFIC DIHYDROFLAVONOL 4-REDUCTASE AND USES THEREFOR

BACKGROUND OF THE INVENTION

Technical field of the invention

The present invention relates to modified DFR nucleic acids and encoding the modified DFR that preferentially utilize DHK as a substrate and their uses for genetically altering plants to increase the content of pelargonidin-based pigments in the plants.

Description of the Prior art

Anthocyanins are classes of pigments that determine flower color and plant pigmentation in angiosperm plants. Among anthocyanins, pelargonidin-based pigments confer bric-red/orange color to plants, while cyanidin- and delphinidin-based pigments confer red and violet color each (Holton, et al. Plant Cell 7:1071-1083 (1995); Tanaka, et al. Plant Cell Physiol. 39:1119-1126 (1998)). Different ratio of these pigments confers a wide range of flower color. Many anthocyanin biosynthetic genes have been identified. One of key enzyme in the biosynthetic pathway is dihydroflavonol 4-reductase (DFR). The enzyme converts dihydroflavonols (dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM)) to leucocyanidins. The leucocyanidins are subsequently converted to anthocyanins by other enzymes. The conversion of DHK to DHQ and DHM are catalyzed by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H). Since DFRs in most plants can convert all three dihydroflavonols to leucocyanidins, the ratio of three

classes of anthocyanin pigments are mainly determined by the activity of F3'H and F3'5'H (Holton, et al. Plant Cell 7:1071-1083 (1995)).

Since pelargonidin-based pigments confer the orange color to flowers, the F3'H and F3'5'H activities must be absent for a plant to have orange colored flowers (U.S. patent 5410096). In many plant species, F3'H and F3'5'H are encoded by a multiple genes, thus the mutant lines that lack F3'H and F3'5'H are not easily found. This partially accounts for the rarity of orange-colored flowers in some plant species. Inability to reduce DHK to leucocyanidin by DFR in some species can also cause the lack of orange-colored flower. For example, DFRs from *Petunia* and *Cymbidium* convert DHK to its leucocyanidin very inefficiently, thus these species do not accumulate large ratio of pelargonidin-based anthocyanins even if F3'H and F3'5'H are absent (Gerats, et al. Planta 155:364-368 (1982); Johnson, et al. Plant J. 19:81-85 (1999)). An orange-colored *Petunia* was engineered by introducing a maize *DFR* to a special mutant line of *Petunia* that lacks *F3'H* and *F3'5'H* (Meyer, et al. Nature 330:677-678 (1987)). Since the maize DFR can convert all three dihydroflavonols to their leucocyanidins, such a mutant line that accumulates DHK was necessary for the development of orange-colored *Petunia*. The necessity of the special mutant line can be circumvented by using a DFR that utilizes DHK preferentially over DHQ and DHM.

Using chimeric *DFR*s between *Petunia* and *Gerbera DFR*s, we identified a region that determines the substrate specificity of DFR. By altering an amino acid in the region, we developed a DHK-specific *DFR* that converts DHK preferentially over

DHQ and DHM. When expressed in plants, the DHK-specific *DFR* increases the pelargonidin-based pigments regardless of F3'H activity.

SUMMARY OF THE INVENTION

Accordingly, the object of this invention is to provide substrate-specific *DFR*s which have altered amino acid sequences at the substrate specificity determining region.

It is an also object herein to provide a DHK-specific *DFR* and nucleic acids encoding the DHK-specific *DFR*.

Still further, it is an object herein to provide transgenic plants expressing the DHK-specific *DFR* which confers a phenotype characterized by the increased content of pelargonidin-based pigments in the plants.

In accordance with the objects, the invention includes the modified DFRs and nucleic acids encoding the modified DFRs which have altered amino acid sequences at the substrate specificity determining region. The properties of modified DFRs are characterized by their abilities to reduce one substrate preferentially among DHK, DHQ, and DHM.

The invention also includes a modified DFR that reduces DHK preferentially over DHQ and DHM.

The invention also includes plants having at least one cell transformed with a vector comprising at least a portion of the modified DFR nucleic acids. Such plants have a phenotype characterized by the increased content of pelargonidin-based pigments.

The invention also includes vectors capable of transforming a plant cell to increase the content of pelargonidin-based pigments.

The invention also includes methods for producing plants having the increased content of pelargonidin-based pigments. The methods includes steps of transforming plant cells with vectors containing the modified DFR gene; regenerating plants from the transformed cells and selecting the plant having the increased content of pelargonidin-based-pigments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic diagram showing three chimeric *DFR*s. Black bars indicate sequences from a *Gerbera DFR* and gray bars indicates sequences from a *Petunia DFR*. Numbers are junctional amino acid positions from the translation start site of the *Gerbera DFR*. C.1, C.2, C.3 are the name of three different chimeric *DFR*s.

Figure 1B shows representative flowers of transgenic *Petunia* expressing chimera *DFR*s or control *DFR*. Ger indicate the transgenic flower expressing *Gerbera DFR* and C.1, C.2, and C.3 indicate Chimera 1, Chimera 2, and Chimera 3 each. RL01 line has a functional *Petunia DFR* gene. The C.1 and RL01 bore similar pink

colored flowers while others bore bric-red colored flower.The transgenic W80 flower expressing C.1 has pink color, while transgenic W80 flowers expressing C.2 and C.3 have orange/bric-red color. The orange/bric-red color can be also observed in the transgenic *Petunia* flowers expressing the native *Gerbera DFR*.

Figure 1C shows the TLC analysis data of pigments produced in transgenic *Petunia* flowers next to standard pigments (pelargonidin (Pg), cyanidin (Cy), and delphinidin (Dp)). The transgenic flowers expressing C.1 has mainly cyanidin- and delphinidin-based pigments, while the flowers expressing C.2 and C.3 have mainly pelargonidin-based pigments in addition to small amount of cyanidin- and delphinidin-based pigments.

Figure 2 shows the amino acid sequence of *Gerbera* DFR aligned with other representative DFR sequences. The ClustalW program was used to align multiple amino acid sequences (Thomson, et al. Nucl. Acids Res. 22:4673-4680 (1994)). The substrate specificity determining region is boxed and the 134th amino acid residue of *Gerbera* DFR and coressponding amino acid residues of DFRs from a few representative species are bold typed. .

Figure 3A shows site-directed mutagenesis of substrate specificity determining region. The sequence corresponds to the substrate specificity determining region of *Gerbera* DFR. Arrows and letters indicates amino acids that were changed to.

Figure 3B shows flowers of transgenic *Petunia* expressing mutated *Gerbera DFR* gene. Ger indicates the wild type *Gerbera DFR* and T132V indicates the

mutated *DFR* that has valine instead of threonine at the 132th position of *Gerbera* DFR. Names of other mutated *DFR*s followed the same notation rule. All transgenic lines except N134L and E145L have the same bric red colored flower. The N134L bore slightly different colored flowers and E145L bore white flowers.

Figure 3C shows a TLC analysis of pigments produced in the transgenic *Petunia* flowers. As expected, the E145L did not accumulated any anthocyanin. The N134L accumulated mostly pelargonidin while other mutated *DFR* and wild type Gebera *DFR* accumulated significant amount of cyanidin and delphinidin in addition to perlargonidin.

Figure 4A shows the development of a *DFR* that display the altered substrate specificity. WR and WV indicate *Petunia* lines that are $dfr^{-/-}$, but $F3'H^{+/+}$ (WR) or $F3'5'H^{+/+}$ (WV). The mark – indicates no *DFR* gene, DFR^{N134L} indicates *DFR* that has leucine instead of aspargine at the 134th position of *Gerbera* DFR, and DFR^{WT} indicates the wild type *Gerbera DFR*. The flower located in the cross section indicate the WR or WV transgenic flowers expressing DFR^{N134L} or DFR^{WT}.

Figure 4B shows a TLC analysis of pigments produced in the transgenic lines. Pg, Cy, and Dp indicate pelargonidin, cyanidin, and delphinidin. The WR and WV lines expressing wild type *DFR* accumulated cyanidin and delphinidin each. The WR line expressing DFR^{N134L} accumulated pelargonidin and cyanidin, while the WV line expressing DFR^{N134L} did not accumulated any pigment other than background level of delphinidin.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, the substrate specificity determining region was identified by determining the abilities of three chimeric *DFR*s to catalyze the reduction of DHK in the transgenic *Petunia* lines. In order to identify the region of DFR that determines its substrate specificity, we constructed chimeric *DFR* genes using cDNA sequences of *Petunia* and *Gerbera*. Though these two DFRs have high similiarity at the amino acid level, *Gerbera* DFR is able to catalyze dihydrokaempferol (DHK) while *Petunia* DFR cannot (Elomaa et al.Mol. Gen. Genet. 248:649-656 (1995)). We built three different chimeric genes using regions of high homology as common PCR primer sites (Fig1A). The chimeric genes were transformed into a white flowered *Petunia* mutant (W80) that lacks DFR activity and accumulates primarily DHK but with appreciable amounts of dihydroquercetin (DHQ) and dihydromyricetin (DHM) (Huits et al., 1994). Chimera 1 produced pink flowers while Chimeras 2 and 3 bore orange-pink flowers (Fig. 1B). The hue of Chimera 1 flowers is very similar to the inbred *Petunia* mutant RLO1, which has functional DFR activity and accumulates DHK. Thin layer chromatography (TLC) determined that Chimera 1 produced mainly cyanidin and delphinidin (Fig. 1b). Chimeras 2 and 3 primarily produced pelargonidin (Fig. 1C), which is the downstream product of DFR reduction of DHK. These results indicated that the region of DFR conferring the ability to reduce DHK was between Chimeras 1 and 2. The identified region (approx. 40 amino acids) is highly variable in DFRs from different plant species. By excluding the completely conserved amino acid sequences at the borders, the identified region is narrowed down to 26 amino acids. Hereinafter, this region is referred as substrate specificity determining region. An example of the

substrate specificity determining region in a few representaive DFRs is shown in Figure 2.

The invention provides the modified *DFR* nucleic acids and encoded DFRs that have altered amino acid sequences at the substrate specificity determining region. Such DFRs have properties characterized by the altered substrate specificity. Hereinafter, DFRs that catalyze the reduction of one substrate preferentially over other two substrates are referred as substrate-specific DFRs. In the preferred embodiments, the invention provides the modified DFR that has altered amino acid at 134th amino acid residue of *Gerbera* DFR or the corresponding amino acid residues of DFRs from other species. Such DFRs have properties characterized by catalyzing the reduction of DHK preferentially over DHQ and DHM. Hereinafter, DFRs that catalyze the reduction of DHK preferentially over DHQ and DHM are referred as DHK-specific DFRs. The 134th amino acid residue of *Gerbera* DFR and corresponding amino acid residues of DFRs from a few representative species are shown in Figure 2.

In accordance with the present invention, a DHK-specific DFR was developed by replacing asparagine at 134th amino acid residue of *Gerbera* DFR to leucine. The expression of the DHK-specific DFR in W80 *Petunia* line, which accumulates large amount of DHK in addition to appreciable amount of DHQ and DHM, caused the production of only pelargonidin. The expression of native *Gerbera* DFR in the same *Petunia* line caused the production of appreciable amounts of cyanidin and delphinidin in addition to pelargonidin (Figure 3). Since the W80 *Petunia* line we transformed accumulates mainly DHK with small amount of DHQ and DHM, it was

not clear if the N134L mutant DFR completely lost the capability of reducing DHQ and DHM. To investigate if the N134L mutant DFR produces only pelargonidin in the presence of fully active flavonoid-3'-hydroxylase (F3'H) or flavonoid-3',5'-hydroxylase (F3'5'H), we crossed our N134L transformant with *Petunia* lines that are either *dfr*$^{-/-}$/*F3'H*$^{+/+}$ (WR line) or *dfr*$^{-/-}$/*F3'5'H*$^{+/+}$ (WV line). As shown in figure 4A, both WR and WV lines bore white flowers as expected. When these lines were crossed with the N134L transformants, the WR line expressing the mutant *DFR* (WR/DFR^{N134L}) had orange colored flowers while the WR expressing wild type *Gerbera DFR* (WR/DFR^{WT}) had red colored flowers. Unlike the WR lines, the WV lines expressing the mutant *DFR* (WV/DFR^{N134L}) bore white flowers while WV lines expressing the wild type *DFR* (WV/DFR^{WT}) had violet colored flowers. To determine the pigments produced in these crossed lines, we performed TLC analysis. Figure 4B shows that the WR/DFR^{N134L} accumulated a large amount of pelargonidin while WR/DFR^{WT} mainly accumulated cyanidin. In the white flowered WV/DFR^{N134L}, no appreciable amounts of anthocyanidins accumulated other than a background level of delphinidin. In contrast to WV/DFR^{N134L}, the WV/DFR^{WT} accumulated mainly delphinidin. The data indicate that the N134L mutant DFR preferentially utilizes DHK as a substrate over DHQ and cannot reduce DHM. The substrate preference of the N134L mutant DFR is somewhat opposite to that of *Petunia* DFR which prefer DHM over DHQ and cannot use DHK (Forkmann and Ruhnau, 1987).The results indicates that the DHK-specific DFR can increase the pelargonidin-based pigments in plants regardless of the presence of F3'H activity.

The invention also provides plants having cells transformed with vectors comprising at least a portion of the substrate-specific *DFR* nucleic acids. Such plants

have phenotypes characterized by the increased content of anthocyanins specified by the substrate specific *DFR*s. In the preferred embodiments, the invention provides plants having cells transformed with vectors comprising at least a portion of the DHK-specific *DFR* nucleic acids. Such plants have phenotypes characterized by the increased content of pelargonidin-based pigments. Plants that can be used to practice the invention include plants within the Division of Magnoliphyta, i.e. the angiosperms include the dicotyledons and the monocotyledons. Particularly preferred Orders of angiosperms according to "Plant Systematics", S.B. Jones, Jr. and A.E. Luchsinger include Magnoliales, Laurales, Aristolochiales, Nymphaeales, Ranunculales, Caryophyllales, Malvales, Violales, Capparales, Ericales, Primulales, Rosales, Fabales, Myrtales, Cornales, Rhamnales, Sapindales, Geraniales, Apiales, Gentianales, Solanales, Lamiales, Scrophulariales, Campanulales, Rubiales, Dipsacales, Asterales, Hydrocharitales, Arales, Cyperales, Liliales, and Orchidales. Particularly preferred plants include orchid, iris, campanula, gentiana, phlox, cyclamen, eustoma, crocus, delphinium, ageratum, chrysanthemum, *Petunia*, cactus, limonium, astilbe, carnation, *Gerbera*, brassica, impatience, geranium, dahlia, sunflower, dianthus, gloxinia, caledula, bellis, ranunculus, aster, tagetes, salvia, hibiscus, cirsium, godetia, catharanthus, alyssum, lupinus, portulaca, drotheanthus, tulip, lily, narcissus, freesia, anemone, gladiolus, caladium, archimenes, achillea, agapanthus, aethiones, allium, alstroemeria, amaryllis, anagallis, androsace, anemone, antirrhinum, aquilegia, armeria, asperula, begonia, browallia, callistephus, camellia, ceanothus, chionodoxa, cistus, clarkia, clematis, colchicun, consolida, cornus, cosmos, deutzia, digitalis, erigeron, erodium, erysimum, erythronium, felicia, gazania, gypsophila, helenium, helianthemum, heliophila, hippeastrum, hyacinthus, hydrangea, iberis, ipomoea, ixia, jacaranda, kalmia, kolkwitzia, lagerstroemia, lathyrus, lavatera,

legousia, lewsia, linum, lobelia, lobularia, magnolia, malus, malva, mathiola, merendera, mimulus, myosotis, narcissus, nemesia, nicotiana, nopalxochia, nymphaea, omphalodes, orthrosanthus, osteospermum, oxalis, paeonia, pelargonium, penstemon, pentas, pericallis, persicaria, platycodon, polemonium, polygala, potentilla, primula, prunus, puschkinia, rhododendron, rhodohypoxis, rose, saintpaulia, saponaria, saxifraga, scabiosa, schizostylis, schlumbergera, schilla, sedum, senecio, silene, solanum, spiraea, stachys, streptocarpus, syringa, tagetes, tanacetum, thunbergia, thymus, torenia, tropaeolum, verbena, veronica, viburnum, vinca, viola, vitis, watsonia, and zinnia. The broad applicability of the modified *DFR* nucleic acids is based on the universal function of DFR in anthocyanin biosynthesis in divergent plant taxa. The parent plant used to practice the invention can be a wild type variant, a mutant which has been generated by the mutagenesis, or a transgenic line that has been generated by the recombinant techniques.

The invention also provides plant transformation vectors comprising at least a portion of substrate-specific *DFR* nucleic acids. In the preferred embodiments, the invention provides a plant transformation vector comprising at least a portion of DHK-specific *DFR* nucleic acids. Particularly preferred promoter to drive the expression of the DHK-specific *DFR* nucleic acids is the cauliflower mosaic virus 35S protein promoter. However, other constitutive promoters, tissue specific promoters, or inducible promoters can be also used.

The transformation of plants can be carried out in accordance with the invention by any of various transformation methods known to those skilled in the art of plant molecular biology. Particular methods for transformation include the transfer

of nucleic acids into a plant cell by the microinjection, polyethylene glycol, electroporation, or microbombardment. Alternatively, plant cells can be transformed by Agrobacterium harboring vectors comprising at least a portion of modified *DFR* nucleic acids.

Regeneration of plants from the transformed cells can be carried out by any methods known to those skilled in the art. See, e.g., Methods in Enzymology, supra.; Methods in Enzymology, Vol 118; and Klee et al. Annual Review of Plant Physiology 38:467-486. Transformed cells or plants are selected based on their resistance to certain chemicals such as antibiotics or based on their phenotypes characterized by the increased content of pelargonidin-based pigments. The transformed plants can be self-fertilized or crossed with other plants. After the fertilization, the plants expressing at least portion of the modified *DFR* nucleic acids can be selected based on their resistance to certain chemicals such as antibiotics or based on their phenotypes characterized by the increased content of pelargonidin-based pigments. Alternatively, the transformed cells or a part of transformed plants can be grafted to other plants.

The following is presented as examples and is not to be construed as a limitation on the scope of the invention.

EXAMPLE

Petunia transformation

Leaf explants of the inbred *Petunia* W80 line (*an6*$^-$, *ht1*$^-$, *ht2*$^-$, *hf1*$^-$, *hf2*$^-$, *fl*$^-$, and *rf*$^-$) were transformed as described elsewhere except that leaf explants recently

infected by *Agrobacterium tumefaciens* were rinsed with Murashige-Skoog solution containing 750 mg/L cefotaxime and then placed on media having 100 mg/L kanamycin sulfate and 500 mg/L cefotaxime(Johnson, et al. Plant J. 19:81-85 (1999)). Also, putative transformants were grown on MS media with vitamins, 30 g/L sucrose, 0.6% agar and 500 mg/L cefotaxime; after rooting the transformants were transferred to soil.

Chimeric gene construction

Highly conserved regions of the *DFR* gene were identified by a multiple sequence alignment of a number of DFRs. The 5' region (*Gerbera DFR* portion) of each chimeric gene was synthesized from the *Gerbera DFR* cDNA clone using a primer containing the codon for the starting methionine of the *Gerbera DFR* gene (5'-GGC GAA AAT GGA AGA GGA TTC TCC-3') and a primer containing a conserved region of the *Gerbera DFR* gene (Chimera 1: 5'-AGC AGA TGA AGT GAA CAC TAG TTT CTT CAC-3'; Chimera 2: 5'-GGC TTT CTC TGC CAG AGT TTT TGA CAC GAA-3'; Chimera 3: 5'-GTG GGA CGA GCA AAT GTA TCT TCC TTT TGC-3'). The 3' region (*Petunia DFR* portion) of each chimeric gene was synthesized from the *Petunia DFRA* cDNA clone using a primer complementary to the three conserved regions (Chimera 1: 5'-TTC ACT TCA TCT GCT GGA ACT CTC GAT GTG; Chimera 2: 5'-CTG GCA GAG AAA GCC GCA ATG GAA GAA GCT-3'; Chimera 3: 5'-ATT TGC TCG TCC CAC CAT GCT ATC ATC TAC-3') and a primer containing the stop codon of the *Petunia DFRA* gene (5'-GCG CTA GAC TTC AAC ATT GCT TAA-3'). 5' and 3' regions were gel purified after PCR amplification. To assemble the full length chimeric gene the 5' and 3' region fragments were added to the same tube in roughly equal amounts and subjected to 25

PCR cycles (94°C 30", 55°C 30", 72°C 1:30). Full length chimeric genes (~1.1 kb) were purified from agarose gels. The chimeric genes were cloned into a vector containing the 35S CaMV promoter and NOS terminator. *Pfu* polymerase (Stratagene, La Jolla, CA) was used for all PCR reactions.

Amino acid point mutant construction

Gerbera DFR genes containing one amino acid point mutation were made in a similar manner as the chimeric genes. The 5' region was synthesized using a primer having the *Gerbera DFR* starting methionine and a primer containing a single codon change. The 3' region was made with a complementary primer with the single codon change and a primer having the stop codon of *Gerbera DFR*. The full length mutant sequence was assembled like the chimeric genes above. Each point mutant was cloned into a vector having the 35S CaMV promoter and NOS terminator. The mutagenized region of each mutant *DFR* was sequenced to ensure the correct residue was changed. Point mutants were then transformed into the W80 *Petunia* line. The transformants expressing the *DFR* genes were crossed with WR *Petunia* line ($dfr^{-/-}$, $F3'H^{+/+}$) and WV *Petunia* line ($dfr^{-/-}$, $F3'5'H^{+/+}$) to determine the substrate specificity of the mutated DFR. Mutations in other loci were not determined in these two *Petunia* lines.

TLC analysis

Anthocyanidins were separated on cellulose TLC plates as described (Johnson, et al. Plant J. 19:81-85 (1999)). Corollas were sometimes stored at 4°C for extended periods of time in methanol-0.5% HCl solution. Before adding iso-amylalcohol, the flower extracts were quantified at 530 nm to ensure uniform loading on the TLC

plate. Anthocyanin standards were purchased from Apin Chemicals Ltd. (Oxfordshire, England).

Sequence alignment

Multiple sequence alignment of DFRs was done using ClustalW program.